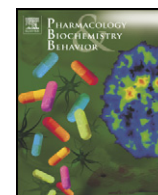


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# Impaired auditory and contextual fear conditioning in soman-exposed rats<sup>☆</sup>

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## ABSTRACT

Exposure to soman (GD) can result in prolonged seizures and subsequent neuropathology in a variety of brain regions including the amygdala and hippocampus. Both regions are believed to play important roles in the development and expression of fear conditioning. The purpose of this experiment was to test these conditioning tasks as a possible behavioral correlate of the observed neuropathology. Male rats were exposed to GD ( $1.0$  or  $1.2 \times LD_{50}$ ) or saline followed with injections of atropine sulfate, the oxime HI-6 and diazepam. Fear conditioning was conducted on post-exposure day (PED) 8 followed by measuring freezing to contextual and auditory conditioned stimuli on PED 9 and 10 respectively. Contextual and auditory fear conditioning was severely impaired in both the  $1.0 \times LD_{50}$  and  $1.2 \times LD_{50}$  GD groups. Both GD groups spent less time freezing than controls when returned to the context in which conditioning occurred. The  $1.0 \times LD_{50}$  and  $1.2 \times LD_{50}$  groups had very low levels of freezing following presentation of the auditory conditioned stimulus. Neuronal fiber degeneration was present in the piriform cortex, thalamus, and amygdala in GD-exposed animals regardless of dose. The present study suggests that contextual and auditory fear conditioning is impaired in GD-exposed rats possibly due to neuropathology observed in the hippocampus, amygdala and thalamus.

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## 1. Introduction

Soman (GD) is a powerful organophosphorous nerve agent that irreversibly binds to cholinesterase enzymes inhibiting the inactivation of the neurotransmitter acetylcholine. The resulting cholinergic hyperactivity is manifested in such symptoms as hypersecretion, cardiovascular dysfunction, respiratory distress, seizures and convulsions. Exposure to high doses of organophosphorus agents induces status epilepticus (SE) in rodents and results in profound neuropathology. Affected regions include the piriform cortex, amygdala, thalamus and hippocampus (Carpentier et al., 1990; Petras, 1994; Shih et al., 2003). Often the resulting neuropathology is associated with impairments in a variety of behaviors. Deficits in various measures of motor function, including grip strength (Filliat et al., 2007; Haggerty et al., 1986), spontaneous locomotor activity (Buccafusco et al., 1990; Haggerty et al., 1986; Landauer and Romano, 1984), and rotor-rod (Filliat et al., 2007; Landauer and Romano, 1984; Romano and Landauer, 1986) are observed following nerve agent exposure. Cognitive impairments often result

from nerve agent exposure. Spatial memory, evaluated by the Morris water maze test, is impaired in rodents exposed to GD (Brandeis et al., 1993; Filliat et al., 2007). Raffaele et al. (1987) found learning deficits in the Stone maze in rats with abnormal brain pathology following GD exposure. Performance in passive (Buccafusco et al., 1990; Choi et al., 2004) and active avoidance (Philippens et al., 1992) tests is poor in GD-exposed rats. Soman exposure also alters the acquisition and maintenance of operant responding in rats (Brezenoff et al., 1985; Harris et al., 1984; Hymowitz et al., 1985, 1990; Modrow and Jaax, 1989).

Pavlovian fear conditioning is a useful procedure often used to elucidate the neural substrates involved in fear-based learning and memory. In this model, a neutral stimulus such as a tone, light or context will serve as a conditioned stimulus (CS) after being paired with an aversive unconditioned stimulus (US) such as foot shock. After conditioning, the rats will freeze in response to the context associated with the US (contextual fear conditioning) or presentations of the CS in a novel environment (auditory fear conditioning). The amygdala and hippocampus have been implicated to play substantial roles in fear conditioning (Goosens and Maren, 2001; Maren et al., 1996; Phillips and LeDoux, 1992; Wilensky et al., 2006). The amygdala and to a lesser extent, the hippocampus, are both areas that can be damaged following nerve agent-induced seizures. We hypothesize that conditioned freezing will be impaired following GD-exposure resulting from damage to the amygdala and hippocampus. The present study focuses on developing a fear conditioning protocol to

<sup>☆</sup> The opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army or the Department of Defense.

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be used in describing the cognitive impairments induced by GD. Once developed, fear conditioning can be used as a functional consequence of observed neuropathology to screen potential neuroprotectants.

## 2. Materials and methods

### 2.1. Subjects

Forty-seven male Sprague–Dawley rats (250–300 g at the start of the experiment) were individually housed and maintained on a reverse light–dark cycle (lights on 21:00–09:00) with laboratory rat chow and water available *ad libitum*. Following surgery and 24 h after GD exposure the rats were fed a wet mash of the laboratory rat chow and sugar. The rats were surgically implanted with a telemetry transmitter (F40-EET, Data Sciences International, Inc.) for the continuous monitoring and collection of electroencephalographic (EEG) activity. Behavioral tests were conducted between 10:00–15:00 and were preceded by a 15- to 30-minute habituation period in a darkened room following transport from the colony room. The experimental protocol was approved by the Animal Care and Use Committee at the U.S. Army Medical Research Institute of Chemical Defense and all procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and the Animal Welfare Act of 1966, as amended. This research was conducted at an AAALAC-accredited facility.

### 2.2. Surgery

The rats were anesthetized with isoflurane (3% induction, 1.5–2% maintenance) and placed in a Kopf (Tujunga, CA) stereotaxic frame. Two sets of cortical stainless-steel screw electrodes were implanted in the skull (A/P, +1.6; L,  $\pm$ 2.0 and A/P, –4.0; L,  $\pm$ 2.0). Insulated stainless steel wires from the F40-EET transmitter implanted subcutaneously were wrapped around the electrodes and secured in place using dental acrylic. The incision sites were sutured and the rats were administered buprenorphine (10.5  $\mu$ g, 0.07 ml, sc) as an analgesic. The rats were given one week to recover prior to nerve agent exposure.

### 2.3. Telemetry equipment

The home cage was placed on a DSI Physiotel Receiver Model RPC-1 in the colony room for EEG acquisition. Data were digitized at 250 Hz, 70 Hz notch filter, and recorded using Dataquest ART™ 4.1 (Acquisition software; Data Sciences International – DSI, Arden Hills, MN). Body temperature and motor activity were recorded via the system described above. Data were collected continuously while the rats were in the colony room 2 to 3 days prior to exposure and throughout the duration of the experiment.

### 2.4. Nerve agent exposure

Following at least 1 week of recovery from surgery, the rats were exposed to GD [110  $\mu$ g/kg ( $1.0 \times LD_{50}$ ) or 132  $\mu$ g/kg ( $1.2 \times LD_{50}$ ), 0.5 or 0.6 ml/kg, sc] or saline (0.5 ml/kg) followed 1 min later with administration of the oxime HI-6 (93.6 mg/kg, 0.5 ml/kg, im) and atropine sulfate (2 mg/kg, 0.5 ml/kg, im). Diazepam (10 mg/kg, sc) was administered 30 min after seizure onset (the appearance of rhythmic high amplitude spikes) or 50 min post-exposure in rats that did not experienced SE. Standard therapy of an oxime, atropine and diazepam was given to maximize survival while still allowing neuropathology to occur. The dose of GD was varied in attempt to induce SE in the greatest number of animals while not impacting survival. Twenty-four hours after GD intoxication the rats were given a wet mash of food pellets and sugar. Subcutaneous injections of saline (3 ml) were administered as needed to prevent dehydration.

### 2.5. Object preference test

On PED 1 the rats were tested in a 1 m  $\times$  1 m plastic open field (San Diego Instruments, San Diego, CA) with a novel object (chew toy) and a familiar object (nylon bone enrichment from the rat's home cage) for a 15 minute test. Motor activity was recorded and analyzed using the Topscan automated tracking software (Clever Systems, Inc., Reston, VA).

### 2.6. Vestibulomotor test (balance beam test)

Prior to exposure, the rats were trained to balance on and ambulate across a brightly lit 1.5 m long  $\times$  2.5 cm wide beam suspended 92 cm above the floor with a dark goal box at the end of the beam. Latency to reach the goal box and ability to maintain balance were recorded. If the rat fell off the beam or did not reach the goal box within 3 min a maximum latency of 3 min was assigned. The balance beam test was conducted on PED 3, 7 and 14.

### 2.7. Rotor-rod

The locomotor coordination of rats after exposure to nerve agent was assessed using the rotor-rod system (San Diego Instruments, San Diego, CA). The rats received 3 training sessions per day for 2 consecutive days. A baseline of locomotor function was assessed on the third day. Over the first 15 s of the trial the rod accelerated to 10 rpm followed by a 5 rpm increase every 15 s to a maximum speed of 30 rpm. The run lasted 75 s or until the rat fell off the rod. Three runs were conducted each session with 2 min between runs. The rats were tested in afternoon sessions consisting of 3 runs per session on PED 3 and 7 using the procedure described above.

### 2.8. Contextual and auditory fear conditioning

#### 2.8.1. Apparatus

The experiments were conducted in chambers (30.5 cm  $\times$  24.1 cm  $\times$  21.0 cm) fitted with stainless steel grid floors (4.8 mm rods, 1.6 cm apart) and contained within sound attenuating cubicles (Med-Associates, St. Albans, VT). The chambers were equipped with a house light, speaker, light source and video camera mounted to the door of the cubicle. The video data were collected and analyzed using Video Freeze software (Med-Associates). The experiments were carried out with the near infrared light source providing the only illumination so as not to impact animal behavior.

#### 2.8.2. Conditioning

Conditioning was conducted on PED 8. At the start of the conditioning trial the rats were placed in the experimental chambers for a 3-minute habituation period. In the conditioning trial a tone (CS; 85 dB, 1 kHz, 10 s) that terminates with a shock (unconditioned stimulus, US; 1.0 mA, 2.0 s) was presented (modified from Goossens and Maren, 2001). The rats were exposed to 15 CS-US pairings with a 60-second stimulus-free period between pairings. Twenty-four hours (PED 9) after the conditioning trial the rats were returned to the chamber (in absence of the tone and foot shock) for 6 min, and freezing behavior was measured in response to the context. Fear conditioning to the CS was conducted 24 h after contextual fear conditioning (48 h after conditioning session; PED 10). The test chamber was altered by inserting a smooth plastic floor over the grid floor and a curved plastic wall insert on the back wall of the chamber creating in effect a novel context. The test chamber and plastic inserts were cleaned with a different cleaner than what was used during conditioning and contextual testing to prevent the contribution of olfactory cues. Following a 3-minute baseline period the rats were presented with 7 tones (85 dB, 1 kHz, 10 s; 60 s between tone presentations) in the absence of foot shock. The change in video-

pixel composition was used to determine a motion index, a quantified measurement of the animal's behavior.

### 2.9. Neuropathology

At the conclusion of the experiment (PED 15) the rats were deeply anesthetized (75 mg/kg, sodium pentobarbital, ip) and perfused through the aorta with phosphate buffered saline, followed by 4% paraformaldehyde. The brains were sectioned (50  $\mu$ m) and silver stained by FD Neurotechnologies (Catonsville, MD) for pathological analysis. Fiber degeneration was evaluated in each animal in eight slides ranging from 0.96 to  $-7.68$  mm from bregma by trained technicians blinded to the experimental condition. Brain regions of interest were evaluated using a ranking system of 0–4 with a score of 4 indicative of severe fiber degeneration and tissue loss within a region. Brain regions were determined using *The Rat Brain in Stereotaxic Coordinates* (Paxinos and Watson, 2005) and the nuclei were grouped anatomically according to *The Rat Nervous System* (Paxinos, 2004). The regions of interest and groupings used are defined below. The midline nuclei refer to the paraventricular thalamic nucleus (PV), intermediodorsal thalamic nucleus (IMD), rhomboid thalamic nucleus (Rh), reunions thalamic nucleus (Re) and the dorsal part of the posterior hypothalamic area (PHD). The intralaminar nuclei include the central medial thalamic nucleus (CM), paracentral thalamic nucleus (PC), centrolateral thalamic nucleus (CL). The mediodorsal thalamic nucleus is composed of lateral (MDL), medial (MDM) and central (MDC) parts. The lateral amygdala nucleus (LA) includes the dorsolateral (LaDL), ventromedial (LaVM) and ventrolateral (LaVL) parts. The lateral division (CeL) and capsular (CeC) part were considered the central nucleus of the amygdala (CeA).

### 2.10. Data analysis

All statistical analyses were performed using either SPSS 16.0 (SPSS Inc.) or GraphPad Prism version 5 (GraphPad Software, Inc.). Latency and duration of the initial seizure were analyzed using a 2-tailed *t* test. Two-way repeated measures ANOVAs were used to analyze body weights, beam and rotor-rod latencies, and freezing behavior over time during cued and contextual fear conditioning tests. Bonferroni posttests were used to determine effects between groups. Statistical analysis was performed using a one-way ANOVA for the distance traveled in the open field and the freezing times for cued and contextual fear conditioning. Tukey's Multiple Comparison Test was used for *post hoc* analysis. Neuropathology scores were analyzed using a two-tailed Mann–Whitney test. Statistical significance is stated as  $p < 0.05$ .

## 3. Results

Forty-seven rats were exposed to saline or GD following implantation of a telemetric transmitter. The transmitter was removed the day after surgery in one saline control rat due to re-opening of the surgical incision and subsequent chewing of the transmitter leads. Following GD injection, all rats displayed signs of cholinergic poisoning, including excessive chewing, salivation, chromodacryorrhea, fasciculation, oral tonus and splayed hind limbs. Seizures were evident in 10/17 of the  $1.0 \times \text{LD}_{50}$  GD rats and 12/14 of the  $1.2 \times \text{LD}_{50}$  GD group (Table 1). Surprisingly, latency to seizure onset was significantly less in rats receiving  $1.0 \times \text{LD}_{50}$  GD than in the  $1.2 \times \text{LD}_{50}$  GD group ( $p < 0.05$ ). The duration of the initial seizure following exposure did not differ between groups. Fig. 1 illustrates the severe weight loss observed in GD-exposed rats that experienced SE and the moderate weight loss in GD rats that did not have a seizure (GD NS). Shortly after exposure, 1 rat from the  $1.0 \times \text{LD}_{50}$  group and 2 from the  $1.2 \times \text{LD}_{50}$  GD group were moribund and euthanized early.

**Table 1**

Comparison of GD-induced toxicity following exposure in two groups of rats. Seizure latency and initial seizure duration are presented as the mean  $\pm$  S.E.M. The one rat with excessive weight loss and the three moribund rats were excluded from the study. \*  $p < 0.05$  between  $1.0$  and  $1.2 \times \text{LD}_{50}$  groups.

	$1.0 \times \text{LD}_{50}$ GD ( $n = 17$ )	$1.2 \times \text{LD}_{50}$ GD ( $n = 14$ )
Rats displaying seizures	10 (58.8%)	12 (85.7%)
Seizure latency (min)	$5.9 \pm 0.5^*$	$10.3 \pm 1.5$
Initial seizure duration (min)	$211.4 \pm 10.6$	$194.5 \pm 10.0$
Moribund following exposure	1	2
Excluded for excessive weight loss	1	0

An additional rat in the  $1.0 \times \text{LD}_{50}$  GD group was excluded from the study due to excessive weight loss ( $\approx 30$  g) prior to the conditioning session.

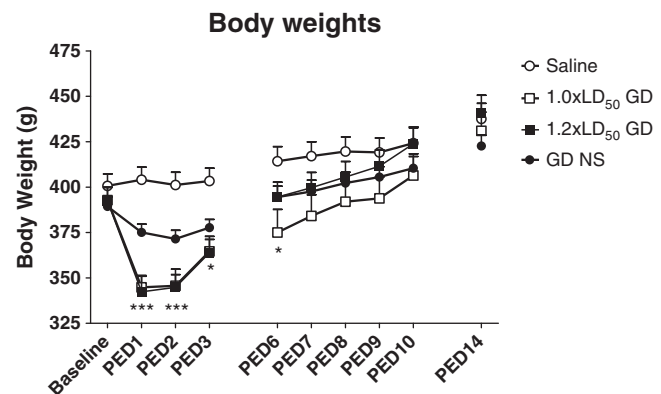
### 3.1. Object preference test

The object preference test conducted on PED 1 revealed limited locomotor activity in both groups of GD-exposed rats compared to controls (Fig. 2). The GD-exposed groups travelled less in the arena [ $F(3,42) = 11.921$ ,  $p < 0.001$ ] compared to saline controls ( $1.0$  and  $1.2 \times \text{LD}_{50}$  GD  $p < 0.001$ ; GD-NS  $p < 0.05$ ); no difference was observed between the two GD-exposed groups. In a similar manner, the speed of travel (data not shown) was reduced in GD-exposed groups [ $F(3,42) = 11.920$ ,  $p < 0.001$ ]. The decrease in total distance traveled at this early time point after exposure was likely due to the overall poor health of the animals. On PED 1 the majority of rats exposed to GD had signs of toxicity, including lethargy, significant weight loss, unkempt fur and, occasionally, recurrent seizures. The interactions with the novel and familiar objects were not analyzed due to the severity of the effects of GD on locomotor activity.

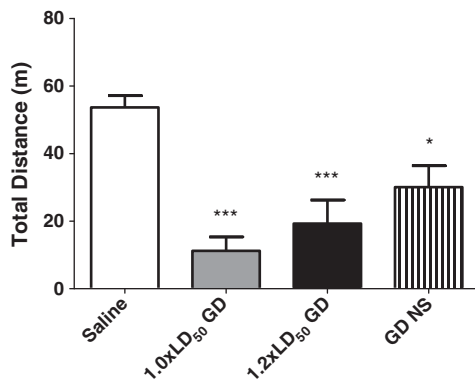
### 3.2. Beam latency

Fig. 3 illustrates the latency to cross the beam and enter the goal box.

GD-exposed groups displayed motor deficits as indicated by an increased latency [Group:  $F(3,39) = 11.94$ ,  $p < 0.001$ ] to reach the goal box at the end of the beam; these deficits were less evident over time [ $F(3, 39) = 10.76$ ,  $p < 0.001$ ]. The motor impairment in GD-exposed rats that experienced SE was most evident at PED 3. GD NS had similar latencies as controls. On PED 3, 70% (7/10) and 83% (10/12) of the  $1.0$  and  $1.2 \times \text{LD}_{50}$  GD groups respectively fell while crossing the beam. Partial recovery was seen by PED 7 when 40% (4/10) of the  $1.0 \times \text{LD}_{50}$



**Fig. 1.** Body weights over time in saline and GD-exposed animals. The rats were weighed daily Monday through Friday at 0800 throughout the duration of the experiment. There was a sharp decline in body weights of the  $1.0$  and  $1.2 \times \text{LD}_{50}$  groups following exposure. The values represent the mean  $\pm$  S.E.M. for each group. \*  $p < 0.05$ , \*\*\*  $p < 0.001$  compared to saline-treated control group.



**Fig. 2.** Total distance traveled in the arena during the object preference test on PED 1 for each group (mean + S.E.M.). \*  $p < 0.05$ , \*\*\*  $p < 0.001$  compared to saline-treated control group.

GD group and 25% (3/12) of the 1.2 × LD<sub>50</sub> GD groups failed to traverse the beam. Only 1 GD NS rat fell once during beam testing (PED 7), which is comparable to baseline testing when one rat from each of the saline, 1.0 and 1.2 × LD<sub>50</sub> GD groups fell during testing. On PED 14 30% (3/10) of the 1.0 × LD<sub>50</sub> GD group fell during testing, while only 8% (1/12) of 1.2 × LD<sub>50</sub> GD rats failed to reach the goal box.

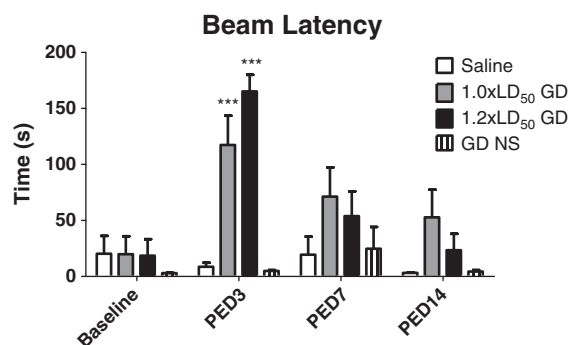
### 3.3. Rotor-rod

Despite obvious motor deficits evident in the balance beam and open field tests, the GD-exposed rats were able to function on the rotor-rod remarkably well. The latency to fall measured prior to exposure and on PED 3 and PED 7 did not differ between or within saline or GD-exposed groups (data not shown).

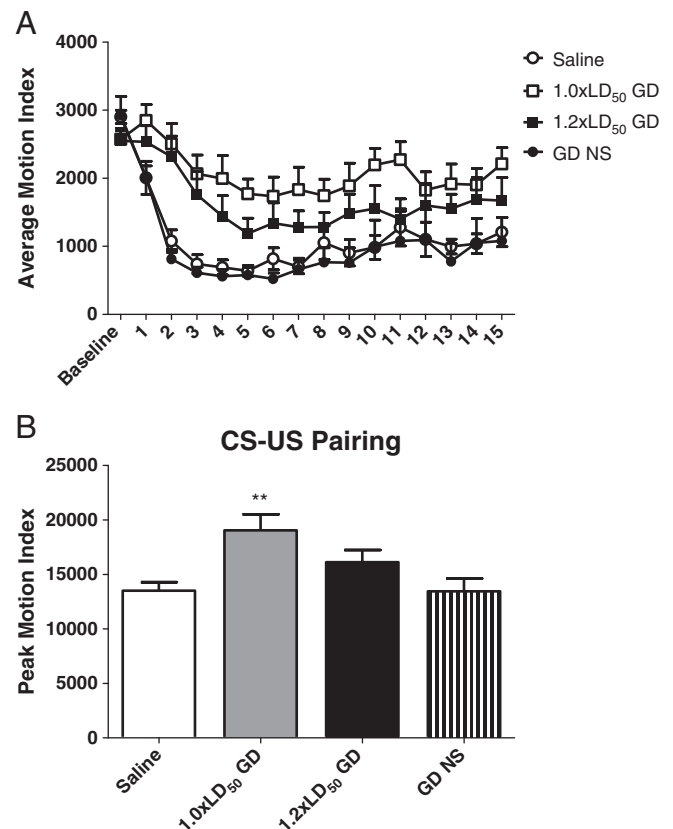
### 3.4. Fear conditioning

#### 3.4.1. Conditioning

At the time of conditioning there were no significant differences in body weight (Fig. 1) or balance beam performance (Fig. 3) between groups. There were no significant differences in activity during the 3-minute baseline period at the start of conditioning (Fig. 4A). One rat from each of the saline, 1.0 × LD<sub>50</sub>, and 1.2 × LD<sub>50</sub> GD groups showed minimal freezing (1.4, 2.5 and 5.6 s respectively) during baseline. The rats exposed to GD showed significantly more activity during the CS–US pairings and the 60 s between pairings than control rats (Fig. 4A). The average peak reaction immediately following the US was compared between groups in Fig. 4B. GD-exposure significantly affected the average peak response [ $F(3,40) = 5.148$ ,  $p < 0.01$ ] with the 1.0 × LD<sub>50</sub> GD group having a greater response to the US than saline controls



**Fig. 3.** Latencies to reach the goal box in the balance beam task prior to exposure and on PEDs 3, 7 and 14. Failure to reach the goal box was assigned a maximal latency of 180 s. The values represent the mean + S.E.M. for each group. \*\*\*  $p < 0.001$ .



**Fig. 4.** Activity during the 15 CS–US pairings in the conditioning session. (A) The average motion index + S.E.M. for each of the CS–US pairings and the 60 s following each pairing (70 s total). (B) The average motion index peak resulting from the US. Rats in the 1.0 × LD<sub>50</sub> group had a greater peak response compared to saline controls. The data is presented as the mean peak response averaged within the session + S.E.M. \*\*  $p < 0.01$ .

( $p < 0.01$ ). There were no significant differences in the total time spent freezing during conditioning (data not shown;  $p = 0.49$ ).

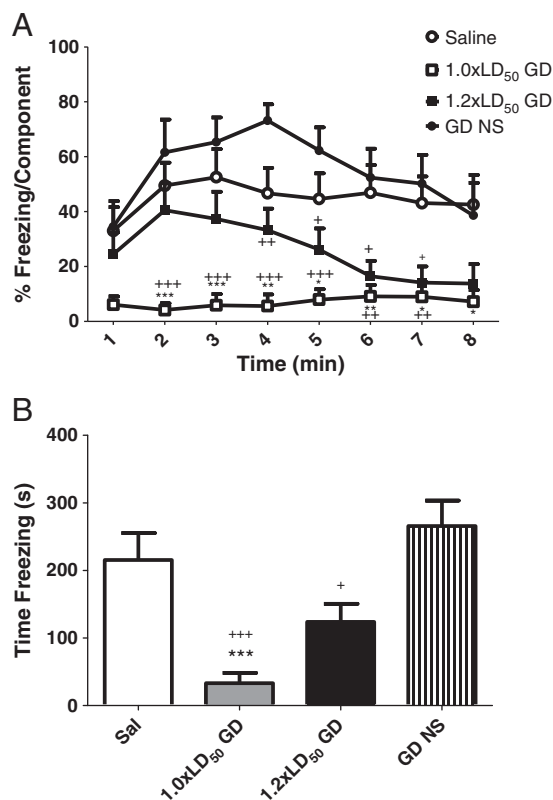
#### 3.4.2. Contextual

Exposure to GD decreased context-induced freezing behavior in the rats (Fig. 5A). There were main effects of both time [ $F(7,30) = 2.345$ ,  $p < 0.05$ ] and exposure [ $F(2,30) = 9.018$ ,  $p < 0.001$ ] and a time/exposure interaction [ $F(14,30) = 1.831$ ,  $p < 0.05$ ]. During context testing, the amount of freezing was less in the 1.0 × LD<sub>50</sub> GD group froze than the saline group during minutes 2–8. Early in the session the 1.2 × LD<sub>50</sub> GD rats spent a similar percentage of the time freezing as saline controls. The only statistically significant time point occurred at minute 6 ( $p < 0.05$ ) when the 1.2 × LD<sub>50</sub> GD rats showed low levels of freezing similar to the 1.0 × LD<sub>50</sub> GD rats. GD exposure also had a significant effect on the total time spent freezing [ $F(3,37) = 7.971$ ,  $p < 0.05$ ] (Fig. 5B). GD NS rats behaved similarly to saline-treated control rats freezing more than either the 1.0 ( $p < 0.001$ ) or 1.2 × LD<sub>50</sub> GD ( $p < 0.05$ ) groups. Of the GD-exposed groups, the 1.0 × LD<sub>50</sub> GD group showed less conditioned freezing than saline controls ( $p < 0.001$ ) while the 1.2 × LD<sub>50</sub> GD group showed a trend toward less freezing than controls ( $p < 0.08$ ).

#### 3.4.3. Auditory

The percentage of time freezing in the novel environment during the 3 minute baseline period differed between groups [ $F(3,38) = 3.73$ ,  $p < 0.05$ ]. Saline ( $14.38 \pm 5.7\%$ ), GD NS ( $19.71 \pm 7.97\%$ ) and 1.2 × LD<sub>50</sub> GD ( $3.10 \pm 2.10\%$ ) rats showed some freezing to the novel environment while 1.0 × LD<sub>50</sub> GD rats did not freeze at all (Fig. 6A). Rats exposed to GD that experienced SE showed impaired freezing behavior when



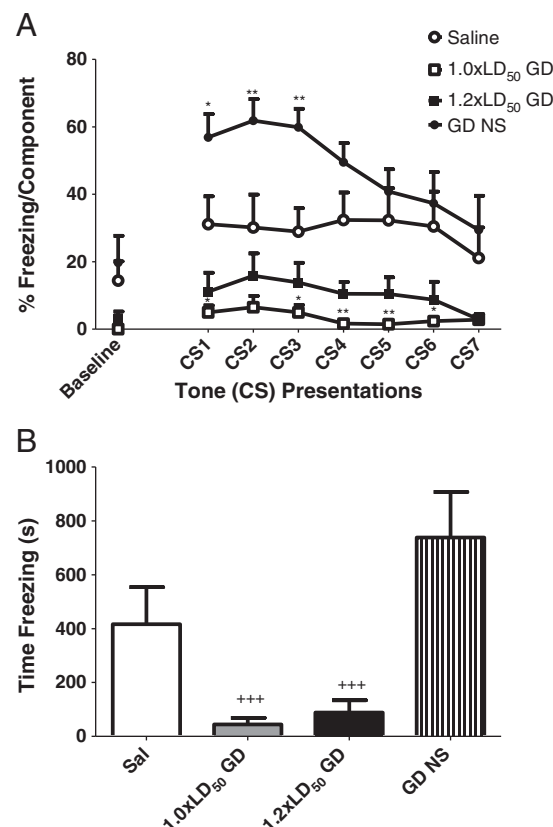


**Fig. 5.** Percent freezing per minute for the 8-minute session in the original context. (A) Rats that experienced GD-induced SE showed impaired response (lack of freezing) across time to a context associated with an aversive stimulus ( $p < 0.001$ ). The values represent the mean  $\pm$  S.E.M. for each group. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared to the control group. +  $p < 0.05$ , ++  $p < 0.01$ , +++  $p < 0.001$  compared to the GD NS group. (B) The total time spent freezing was less in GD-exposed rats compared to controls. Rats exposed to GD (1.0 or 1.2  $\times$  LD<sub>50</sub>) that did not experience SE behaved in a similar manner to saline controls. The data are presented as the total time spent freezing during the 8-minute session averaged for each group  $\pm$  S.E.M. \*\*\*  $p < 0.001$  compared to controls. +  $p < 0.05$ , +++  $p < 0.001$  compared to GD-exposed rats that did not experience SE.

presented with the CS in a novel environment [ $F(3,37) = 15.73$ ,  $p < 0.001$ ] (Fig. 6A + B). Freezing behavior was affected with repeated CS presentations [ $F(6,37) = 5.62$ ,  $p < 0.001$ ] with decreases in both the saline and GD-NS groups with repeated presentations. Surprisingly, the GD NS group froze a greater percentage of the time during the first three CS presentations ( $p < 0.05$ – $0.01$ ) than saline rats. Both the 1.0 and 1.2  $\times$  LD<sub>50</sub> GD froze less than the saline group but only the 1.0  $\times$  LD<sub>50</sub> GD group reached significance at CS1, CS3, CS4, CS5 and CS6 ( $p < 0.05$ – $0.01$ ). GD exposure also had a significant effect on the total time spent freezing [ $F(3,38) = 8.73$ ,  $p < 0.001$ ] (Fig. 4B). Amongst the rats exposed to GD, the GD NS spent more time freezing than both groups of rats that had seizures (1.0  $\times$  LD<sub>50</sub> GD,  $p < 0.001$ ; 1.2  $\times$  LD<sub>50</sub> GD,  $p < 0.05$ ).

### 3.5. Neuropathology

Neuronal tract degeneration was present in the thalamus, piriform cortex, and amygdala and to a lesser extent the hippocampus (Table 2) in GD-exposed animals that experienced SE. There was no evidence of neuropathology in saline control or GD-NS rats. Fig. 7 depicts representative silver stained coronal slices from a control and GD rats from each dose that experienced SE. Neuropathology scores were similar between the 1.0 and 1.2  $\times$  LD<sub>50</sub> GD groups that experienced SE with the exception of greater damage observed in the lateral amygdala and the intralaminar nuclei of the thalamus in the 1.0  $\times$  LD<sub>50</sub> GD group ( $p < 0.05$  for all regions). Extensive degeneration was apparent in the piriform, anterior insular and perirhinal



**Fig. 6.** Percent freezing per CS component (10 s of tone followed by a 60-second stimulus free period) in the novel context. (A) GD-exposed animals that experienced SE showed low levels of freezing throughout the 7 presentations of the CS. The GD-exposed rats that did not experience SE showed an enhanced freezing response to the CS compared to controls. (B) The total time spent freezing throughout the CS presentations. The values represent the mean  $\pm$  S.E.M. for each group. \*  $p < 0.05$ , \*\*  $p < 0.01$  compared to the control group. +++  $p < 0.001$  compared to GD-exposed rats that did not display SE.

cortices, the dorsal endopiriform nucleus, regions of the extended amygdala and the lateral geniculate nucleus (LG), supragenulate nucleus (SG), medial region of the medial geniculate nucleus (MGM), mediodorsal nucleus (MD), intralaminar nuclei (CM, PC and CL) and midline nuclei (PVP, IMD, Rh, Re and PHD) of the thalamus in the rats that experienced SE (Figs. 8–9). Less neuronal degeneration was present in the ectorhinal and endorhinal cortices, and the central, basomedial and medial nuclei of the amygdala.

### 4. Discussion

In the current study deficits in both contextual and cued fear conditioning were observed in both sets of animals exposed to GD regardless of dose; however, deficits occurred only in the animals that had seizures. Fear conditioning was conducted after there were no longer observable motor impairments; eliminating the possibility that the deficits in fear conditioning could be accounted for motor impairments. GD has been reported to have antinociceptive effects in rodents, as measured by the tail flick and hot plate tests (Clement and Copeman, 1984; Haggerty et al., 1986; Romano et al., 1985; Shih and Romano, 1988) although differences in nociception seem unlikely. In a previous experiment latency to lick the hind paw was measured in male Sprague–Dawley rats exposed to GD (1.0  $\times$  LD<sub>50</sub>) on PED 8 using the hot plate test (Lumley, unpublished data). Rats exposed to GD had similar latencies ( $n = 3$ ,  $16.5 \pm 6.8$  s) as the saline-treated controls ( $n = 4$ ;  $16.5 \pm 2.9$  s), suggesting that at the time of fear conditioning (PED 8) there were no significant differences in

**Table 2**

Scored neuronal tract degeneration in rats with GD-induced SE. Brain regions of interest were evaluated using a ranking system of 0–4 with a score of 4 indicative of severe fiber degeneration and tissue loss within a region. No degeneration was evident in control animals or GD-exposed rats that did experience SE. The data are presented as the mean (S.E.M). BLA, basolateral nucleus, BMA, basomedial nucleus; CeA, central nucleus; LA, lateral nucleus; LGN, lateral geniculate nucleus; MD, mediodorsal thalamic nucleus; MGM, medial geniculate nucleus, medial region; Po, posterior thalamic nuclear group; PoDG, polymorph layer of the dentate gyrus; SG, supragenicular nucleus. \*  $p < 0.05$  between  $1.0 \times LD_{50}$  and  $1.2 \times LD_{50}$  groups.

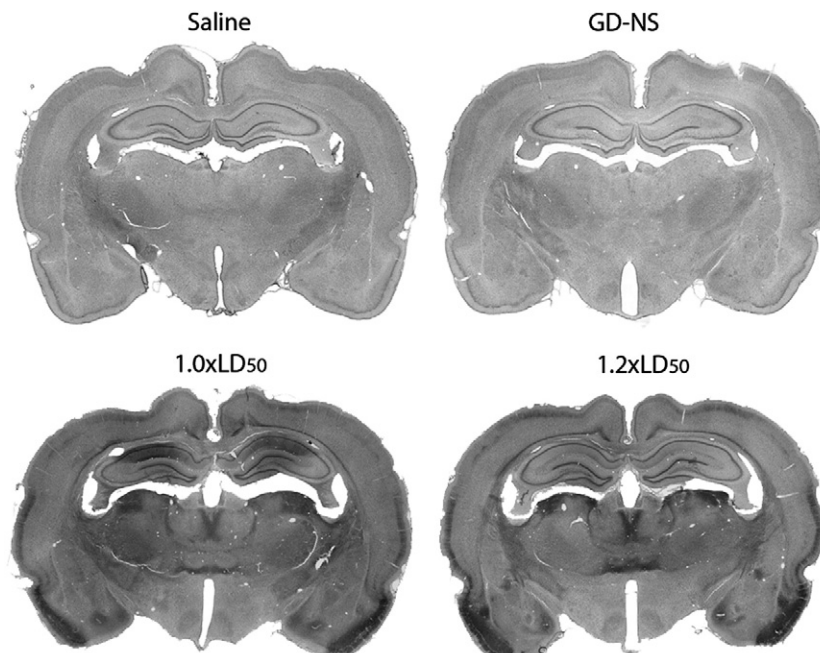
Region	$1.0 \times LD_{50}$ GD	$1.2 \times LD_{50}$ GD
Amygdala		
BLA	$1.9 \pm 0.3$	$1.8 \pm 0.3$
LA	$2.4 \pm 0.3^*$	$1.6 \pm 0.2$
BMA	$1.5 \pm 0.5$	$2.1 \pm 0.4$
CeA	$2.0 \pm 0.4$	$1.8 \pm 0.3$
Cortex		
Auditory	$1.5 \pm 0.4$	$1.7 \pm 0.3$
Ectorhinal	$2.0 \pm 0.4$	$1.8 \pm 0.3$
Insular	$2.2 \pm 0.5$	$2.6 \pm 0.4$
Perirhinal	$1.9 \pm 0.4$	$2.4 \pm 0.4$
Piriform	$3.3 \pm 0.4$	$3.0 \pm 0.3$
Hippocampal formation		
CA1	$2.3 \pm 0.4$	$1.2 \pm 0.4$
CA2	$1.4 \pm 0.5$	$< 1$
PoDG	$1.6 \pm 0.4$	$< 1$
Thalamus		
LGN	$3.4 \pm 0.2$	$2.4 \pm 0.4$
Po	$1.3 \pm 0.6$	$1.4 \pm 0.5$
MGM	$2.4 \pm 0.3$	$2.3 \pm 0.4$
SG	$3.1 \pm 0.3$	$3.3 \pm 0.4$
MD	$2.6 \pm 0.4$	$2.2 \pm 0.4$
Intralaminar	$2.5 \pm 0.4^*$	$1.4 \pm 0.4$
Midline	$3.5 \pm 0.2$	$3.3 \pm 0.2$

nociception. The peak response following the foot shock during conditioning was greatest in the  $1.0 \times LD_{50}$  rats and no different from saline rats in the  $1.2 \times LD_{50}$  group demonstrating that GD-exposed rats that had experienced SE had a greater than or equal response to the foot shock. The observed differences in conditioned freezing are most likely a result of GD-exposure on cognitive functioning as opposed to the effect of GD on locomotor activity or nociception. The impact of GD on contextual and auditory fear conditioning in rats is a

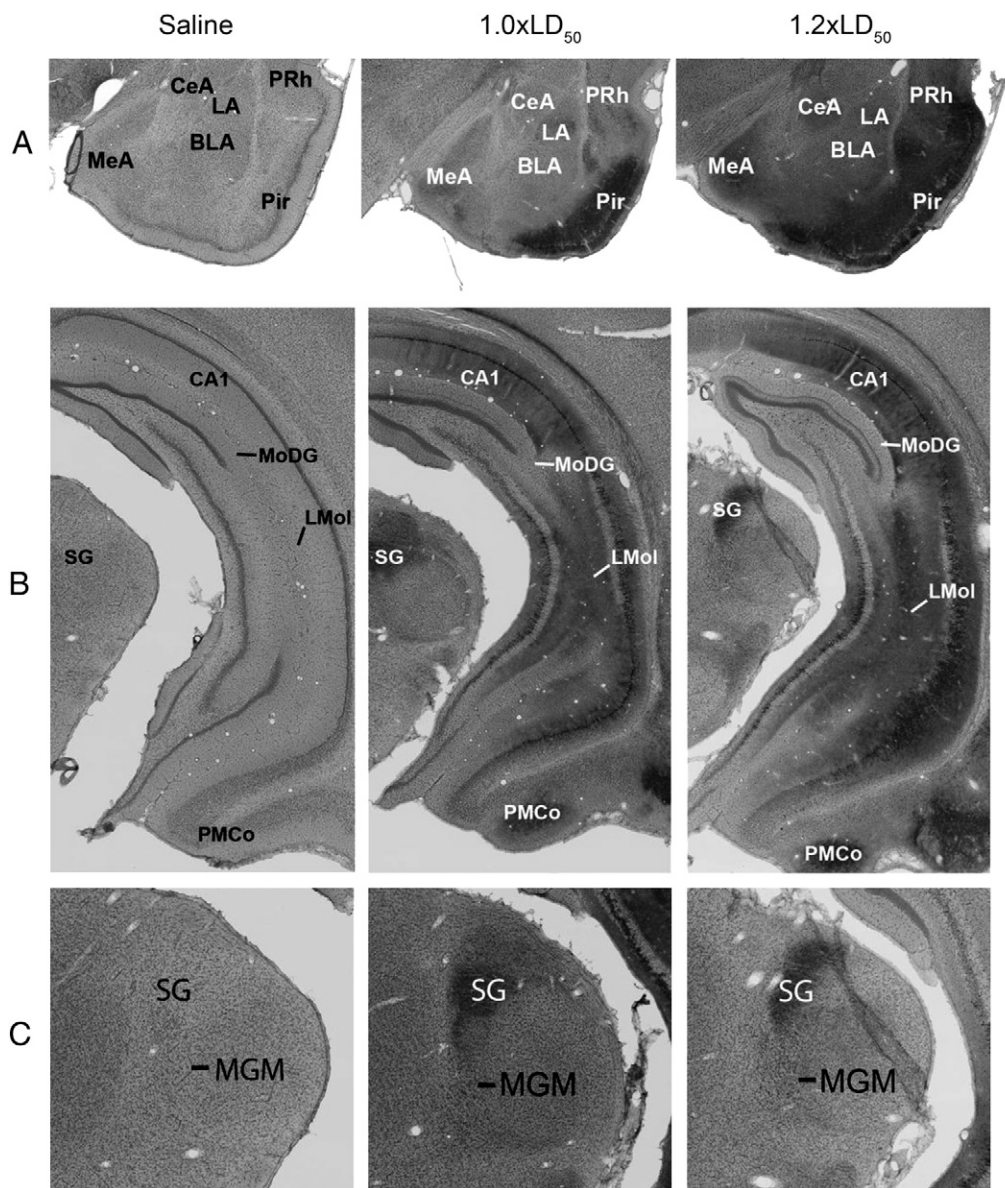
novel finding and differs from previously published studies using animal models of GD-exposure.

There are currently two published studies on the effects of GD on fear conditioning in rodents to the best of our knowledge. [Coubard et al. \(2008\)](#) found at 30 days, but not 90 days following exposure, mice exposed to GD ( $1.2 \times LD_{50}$ ) froze to a greater extent than saline controls in both auditory and contextual fear conditioning. In a companion paper the authors described neuronal death and degeneration in the amygdala occurring at the same time points and dose as in the behavioral study ([Collombet et al., 2008](#)). Both studies included only mice exhibiting physical signs of convulsions following exposure. Atropine methyl nitrate was administered to reduce peripheral cholinergic toxicity but leave the central nervous system unprotected, whereas atropine sulfate was used in this study to protect both the CNS and the periphery. In addition, the oxime HI-6 and diazepam were administered to reduce GD-induced neurotoxicity in the current study. The authors reported profound neuropathology in the amygdala and hippocampus, whereas in the current study the piriform cortex and various thalamic nuclei were the brain regions predominantly affected and only mild damage was observed in the amygdala and hippocampus. The differences in species tested, time of conditioning and pattern of neurotoxicity could account for the differences in fear conditioning. In the second study [Pernot et al. \(2009\)](#) demonstrated impaired contextual but not cued fear conditioning measured 60 days post-injection in mice administered intrahippocampal injections of GD. The intrahippocampal injections of GD did not result in initial seizures but rather had an epileptogenic effect with spontaneous seizures occurring 1–4 weeks post-exposure. Interestingly, the authors report no evidence of neuropathology despite describing impairment in contextual fear conditioning.

Impairments in both spatial and declarative memory are also seen in rat models of SE. Context-induced freezing is reduced in rats with recurrent seizures induced by pilocarpine ([Cardoso et al., 2009](#); [Dos Santos et al., 2005](#); [Szyndler et al., 2005](#)) and kainic acid ([Kemppainen et al., 2006](#)). The few manuscripts examining cued fear conditioning using the pilocarpine model have had mixed results. Rats experiencing SE were found to be severely impaired in auditory fear conditioning ([Dos Santos et al., 2005](#)). In another study, [Cardoso et al. \(2009\)](#) found no differences in freezing responses in SE rats. Differences in



**Fig. 7.** Representative neuropathology following GD-exposure in (A) saline control, (B)  $1.0 \times LD_{50}$ , (C)  $1.2 \times LD_{50}$  GD-exposed rats on PED 14. Silver stained coronal slice at  $-3.00$  mm from bregma.



**Fig. 8.** Silver stained coronal slices showing neuronal fiber degeneration in A) the amygdala and piriform cortex (approximately  $-3.00$  mm from bregma) B) hippocampus (approximately  $-5.52$  mm from bregma) and C) the suprageniculate and medial part of the medial geniculate thalamic nuclei (approximately  $-5.52$  mm from bregma) in saline,  $1.0 \times LD_{50}$  and  $1.2 \times LD_{50}$  GD-exposed rats on PED 14. There was no fiber degeneration evident in the GD-NS group (not shown). BLA, basolateral amygdala; CA1, field CA1 of the hippocampus; CeA, central amygdaloid nucleus; LMol, lacunosum molecular layer of the hippocampus; MGM, medial geniculate nucleus, medial part; MeA, medial amygdaloid nucleus; Pir, piriform cortex; PMCo, posteromedial cortical amygdaloid nucleus; PRh, perirhinal cortex; SG, suprageniculate thalamic nucleus.

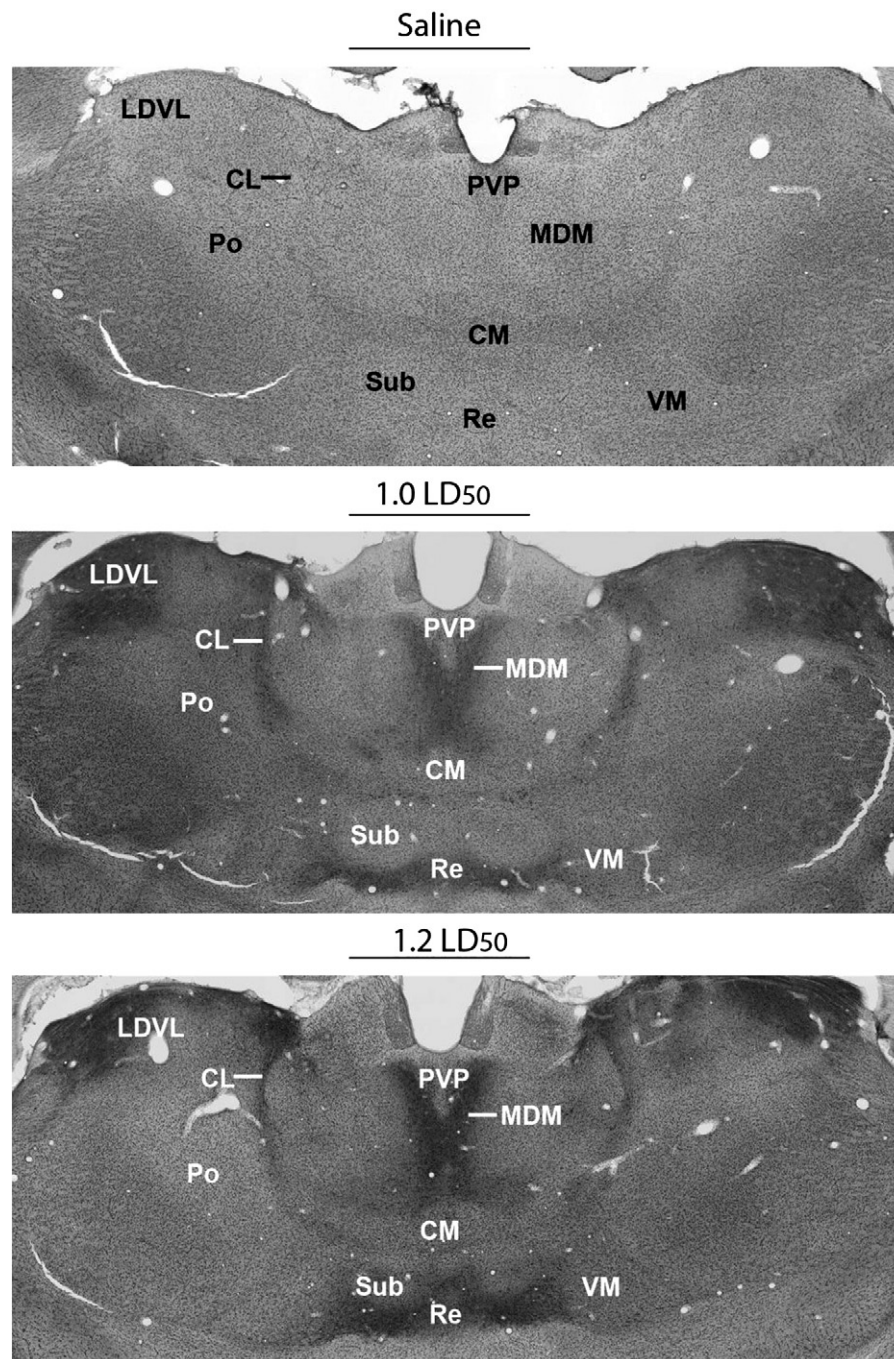
conditioning and testing procedures between the two studies could account for the different results. In addition, strain differences in cognitive functions using the pilocarpine model of SE have been reported (Hort et al., 2000). Cued fear conditioning was not impaired in the kainic acid or electrical stimulated amygdala models of temporal lobe epilepsy (Kempainen et al., 2006).

The enhanced freezing response during the first four CS presentations by GD-NS animals was an unexpected finding. Enhancement of ACh neurotransmission by the inhibition of acetylcholinesterase (AChE) could possibly explain the heightened freezing response in the GD-NS. Although measurement of inhibition of AChE activity over time was beyond the scope of the current study, others have reported persistent GD-induced inhibition of brain AChE over one week post-exposure (Grubic et al., 1981; Lemerrier et al., 1983; Lintern et al., 1998; Tripathi and Dewey, 1989) including HI-6 treated animals (Clement et al., 1991). Pharmacological modulation of cholinergic neurotransmission can have a profound effect on various aspects of

learning and memory (reviewed in Hasselmo, 2006). Administration of the AChE inhibitor physostigmine ameliorates deficits in behavioral tasks of learning and memory in animal models of diencephalic amnesia (Roland et al., 2008), schizophrenia (Cernansky et al., 2005), Alzheimer's disease (Dong et al., 2005) and hypoxia (Bekker et al., 2007; Muthuraju et al., 2009). Physostigmine and physostigmine analogs also improve performance on tasks used to evaluate learning and memory in normal subjects (Brufani et al., 1987; Fitzgerald et al., 1988; Santucci et al., 1989).

Fear conditioning is often used as a behavioral measure of hippocampus and amygdala function. Historically, contextual fear conditioning was thought to be dependent on the hippocampus, whereas both auditory and contextual fear conditioning are influenced by the amygdala (Goosens and Maren, 2001; LeDoux et al., 1988, 1990; Phillips and LeDoux, 1992). The lateral nucleus (LA) of the amygdala plays a critical role in the acquisition and expression of fear conditioning by receiving convergent CS and US sensory inputs





**Fig. 9.** Silver stained coronal slices from approximately  $-3.00$  mm from bregma showing neuronal tract degeneration in thalamic nuclei. Representative slices are taken from saline,  $1.0 \times LD_{50}$  GD and  $1.2 \times LD_{50}$  GD rats. There was no damage in GD-NS rats (data not shown). CL, centrolateral thalamic nucleus; CM, central medial thalamic nucleus; LDVL, laterodorsal thalamic nucleus, ventrolateral part; MDM, mediodorsal thalamic nucleus, medial part; Po, posterior thalamic nucleus group; PVP, paraventricular thalamic nucleus, posterior part; Re, reunions thalamic nucleus; Sub, submedius thalamic nucleus; VM, ventromedial thalamic nucleus.

(Amorapanth et al., 2000; Blair et al., 2005; Campeau and Davis, 1995). The CeA acts as the main output center of the amygdala and projects to motor areas responsible for the conditioned fear response (LeDoux et al., 1988). Recent studies have demonstrated the importance of both the LA and CeA in the acquisition of fear conditioning (Nader et al., 2001; Wilensky et al., 2006). We hypothesized that the neuropathology present in the hippocampus and/or amygdala resulting from GD-induced seizures would disrupt fear conditioning.

Significant GD-induced fiber degeneration was evident in rats that had experienced SE; however, it does not offer a clear explanation of

the deficits in fear conditioning. Consistent but mild damage was seen in various nuclei of the amygdala while hippocampal toxicity was more variable. Mild degeneration was observed in both the LA and CeA; however, the greatest neuropathology was observed in the thalamus and piriform cortex. The LA receives inputs from the thalamus including areas damaged following GD-exposure such as the intralaminar and midline nuclei (RE, PV, CM, CL) as well as the MG (De Olmos et al., 2004). Transmission of the auditory CS to the LA is dependent on projections originating from auditory processing area of the thalamus. Direct projections originate from the MGM, SG and posterior intralaminar thalamic nucleus (direct projections), while those areas

and the dorsal and ventral areas of the MG project to the cortex, which in turn projects to the LA (indirect projections). Disruption of both circuits, but not either individually, prevents auditory fear conditioning in rats (Romanski and LeDoux, 1992). Evidence also exists that the auditory thalamus, especially the MGM and posterior intralaminar nucleus, is an important site of plasticity and plays a more critical role in auditory fear conditioning than just sensory relays (Han et al., 2008; Parsons et al., 2006, reviewed in Weinberger, 2010). Neuropathology was present in the MGM and SG, potentially disrupting the transmission of auditory information to the LA via direct projections or directly disrupting the formation of auditory fear memories. Although damage was not evident in the dorsal or ventral MG, neuropathology was present in the perirhinal cortex, a target of the auditory thalamus inputs and origin of projections to the LA (Witter and Amaral, 2004).

The MD region of the thalamus was damaged in the GD-exposed rats that experienced SE. The MD is often damaged in animal models of temporal lobe epilepsy and is suggested to be involved in the propagation of seizure activity (Bertram et al., 2001, 2008), making it a likely region to be affected by GD-induced seizures. The involvement of the MD in contextual fear conditioning is supported by both human and animal studies. Contextual fear conditioning in rats is attenuated by pre- or post-training lesions of the midline thalamic region including the MD (Li et al., 2004). In addition, the MD is activated in humans showing a conditioned response when presented a context associated with a foot shock (Alvarez et al., 2008).

The determination of the exact mechanism of GD-induced disruption of fear conditioning is beyond the scope of this study. Observed neuropathology was widespread, affecting cortical and subcortical regions. Neuronal degeneration in the thalamus, amygdala or hippocampus could have contributed to the overall behavioral effect observed. Seizures and the subsequent neuropathology appeared to be the determining factor in the appearance of fear conditioning deficits. Myhrer et al. (2005) found cognitive deficits in rats with soman-induced neuropathology, as evidenced by poor performance in a novelty test and retention of a brightness discrimination task. No cognitive impairments were observed in GD-exposed rats that did not convulse. Despite similar seizure durations and neuropathology scores in most regions evaluated the  $1.2 \times LD_{50}$  group often showed less impairment than the  $1.0 \times LD_{50}$  group. A higher percentage of rats receiving  $1.2 \times LD_{50}$  GD (85.7%) experienced seizures than did rats receiving  $1.0 \times LD_{50}$  GD (58.8%). The lower percentage of rats displayed seizures in response to the  $1.0 \times LD_{50}$  GD dose may represent a population that is “sensitive” to the effects of GD requiring a lower dose to induce seizures. The greater variation in the behavioral measures of the higher dose group may be accounted for by the majority of rats experiencing seizures, representing a combined population of rats that include less “sensitive” rats as well as rats with higher toxicity thresholds. The differences in individual animals’ susceptibility to soman and the resulting variations in behavior have been reported previously (Haggerty et al., 1986).

The current results are in agreement with the learning and memory deficits observed in other animal models of SE. The only other manuscript on the effects of systemic nerve agent on fear conditioning found contrary findings, although the differences in species, time span and neuropathology may account for the discrepancy. The observed neuropathology in the amygdala and hippocampus was not as severe as expected. The CeA was mostly spared and only slight damage was noted in LA and hippocampus; however severe fiber tract degeneration occurred in thalamus including nuclei important in sensory processing. The widespread neurodegeneration observed could account for the impairments in fear conditioning. Further studies evaluating the time course of events are warranted. The ease of testing and the magnitude of GD-induced effects make fear conditioning a useful behavioral test for the evaluation of neuroprotectants following GD exposure.

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